EFFECTS OF VARIOUS FACTORS ON THE SYNTHESIS OF ASCORBIC ACID BY THE AMERICAN COCKROACH, PERIPLANETA AMERICANA L.[1]

By P. Gerald Rousell

St. Francis Xavier University, Antigonish, Nova Scotia.

Ascorbic acid has been found in the tissues of a number of insects. Joly (1940) reported it in the blood of the queen termite, *Bellicositermes natalensis.* Haydak and Vivino (1943) showed that the adult honeybee, *Apis mellifica,* contains an average of 1.88 micrograms of the vitamin per gram of tissue. In the aphid *Myzodes persicae,* Barmstedt (1948) found ascorbic acid in the epithelium of the posterior, but not in the anterior mid-gut or in the hind-gut. Metcalf (1943) demonstrated the presence in large amounts of ascorbic acid in the Malpighian tubules of the cockroach, *Periplaneta americana.* Gamo (1941) reported that ascorbic acid is exceedingly important in the development and metamorphosis of the silkworm, *Bombyx mori.* The growth of the larva depends chiefly upon the content of the vitamin in the mulberry leaves upon which it feeds. Day (1949) using a histochemical method, found a few granules in the larva, but none in the adult, of *Tenebrio molitor.*

Wollman, Giroud and Ratismananga (1937) have presented indirect evidence that the cockroach, *Blattella germanica,* is able to synthesize ascorbic acid. They raised cockroaches under aseptic conditions on a vitamin C-free diet for fifteen years and found that the content of ascorbic acid was the same as that in specimens which were not raised in this manner and which were fed a diet containing ascorbic acid.

Gamo and Seki (1954) presented direct proof for the ability of the insect to synthesize ascorbic acid. They demonstrated that homogenates of pupal fat bodies of the silkworm, *B. mori,* were

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able to convert mannose into vitamin C. This ability varied considerably during the period from the prepupa to the four-day pupa at 25° C.

The purpose of the present work is to determine whether homogenates of the cockroach, *P. americana,* are able to synthesize ascorbic acid and the optimal conditions for this synthesis.

MATERIALS AND METHODS

The cockroaches used in all experiments were raised in the laboratory on a diet of dog pellets and water.

The method for ascorbic acid determination was that of Roe and Kuether (1943) as modified by Schaffert and Kingsley (1955), except that only 1 ml. of filtrate was used in each determination, and the amounts of the other reagents were adjusted accordingly. The insects were immobilized with ether and then weighed. Homogenates of whole nymphs were made using a 0.3M phosphate buffer adjusted to the proper pH. One milliliter of the homogenate was then shaken with 3.5 ml. of the substrate in a 15 ml. centrifuge tube and incubated for the desired period of time. A blank consisting of 3.5 ml. of the substrate solution and 1 ml. of buffer was used to compensate for any colored compounds that might be formed by the reaction of the substrate with the reagents. A third tube was used which contained 1 ml. of the homogenate to which 3.5 ml. of the substrate were added at the end of the incubation period. The purpose of this blank was to obtain the amount of ascorbic acid in the homogenate. When this value was subtracted from the reading obtained for the incubated homogenate, the amount of ascorbic acid synthesized was obtained. The three tubes (the tubes containing the reaction mixture, the homogenate blank, and the substrate blank) were incubated at a given temperature for the desired period of time. Readings were made with a Beckman DU spectrophotometer at a wave length of 520 mμ, and a slit width of 0.03 millimeters.

The following factors were studied, and in these tests, mannose was used as the substrate except where others are specified.

(1) The nature of the substrate. Substrates used were mannose, glucose, sucrose, galactose, fructose and xylose.

(2) The effect of substrate concentration. Values tested were 10^{-3}, 3×10^{-3}, 5×10^{-3} and 10^{-2}M.

(3) The effect of homogenate concentration using 10, 20 and 30 per cent.
(4) The length of the incubation period. Periods tested were 1, 2, 3, 6, 9 and 14 hours.
(5) The effect of various pH values. Tests were made using solutions adjusted to 4.4, 5.6, 6.8, 7.4, 8.4. The pH values were determined by means of a Beckman pH meter.
(6) The effect of various incubation temperatures. Those tested were room temperature (approximately 25°), 30°, 35°, 40° and 45° C.
(7) The effect of manganese ion. Solutions of 0.01, 0.05 and 0.10 per cent $MnCl_2$, were used.
(8) The effect of using homogenates of fat bodies alone. Ten per cent homogenates at pH 6.8 were incubated for three hours at 25° C. with substrate solutions of 5×10^{-3}M concentration.

OBSERVATIONS

The ability of the cockroach to utilize sugars as precursors of ascorbic acid is evident from the figures given in Table I. When

TABLE I

THE EFFECT OF THE USE OF VARIOUS SUGARS AS SUBSTRATES ON THE SYNTHESIS OF ASCORBIC ACID BY *P. americana*.

Substrate	No. of Tests	Amount synthesized μg./gm. (with Standard Error)
Mannose	60	18.6 ± 0.58
Glucose	10	15.1 ± 1.34
Fructose	10	13.7 ± 2.00
Sucrose	10	11.6 ± 1.77
Galactose	10	14.6 ± 1.40
Xylose	10	7.2 ± 1.70

sucrose was used, an average synthesis of 11.6 μg./gm. was obtained. Mannose was utilized most efficiently, resulting in the formation of 18.6 μg./gm. while xylose was the least effective, producing 7.2. In a few tests, regardless of substrate, negative values were obtained. Table II contains statistical analyses of the results obtained when the different substrates were used. A significant difference exists between the amount of ascorbic acid

TABLE II

PROBABLE SIGNIFICANCE BETWEEN THE AMOUNT OF ASCORBIC ACID SYNTHESIZED FROM DIFFERENT SUBSTRATES*

Substrates Compared	Difference between Means	Standard Error of Difference	Difference / Standard Error of Difference
Mannose & Glucose	2.90	1.43	2.03
Mannose & Fructose	4.30	2.00	2.05
Mannose & Sucrose	6.40	1.77	3.44
Mannose & Galactose	3.60	1.50	2.26
Mannose & Xylose	10.80	1.80	6.00
Xylose & Glucose	7.90	2.17	3.64
Xylose & Fructose	6.50	2.61	2.10
Xylose & Sucrose	4.40	2.48	1.77
Xylose & Galactose	7.20	2.20	3.27

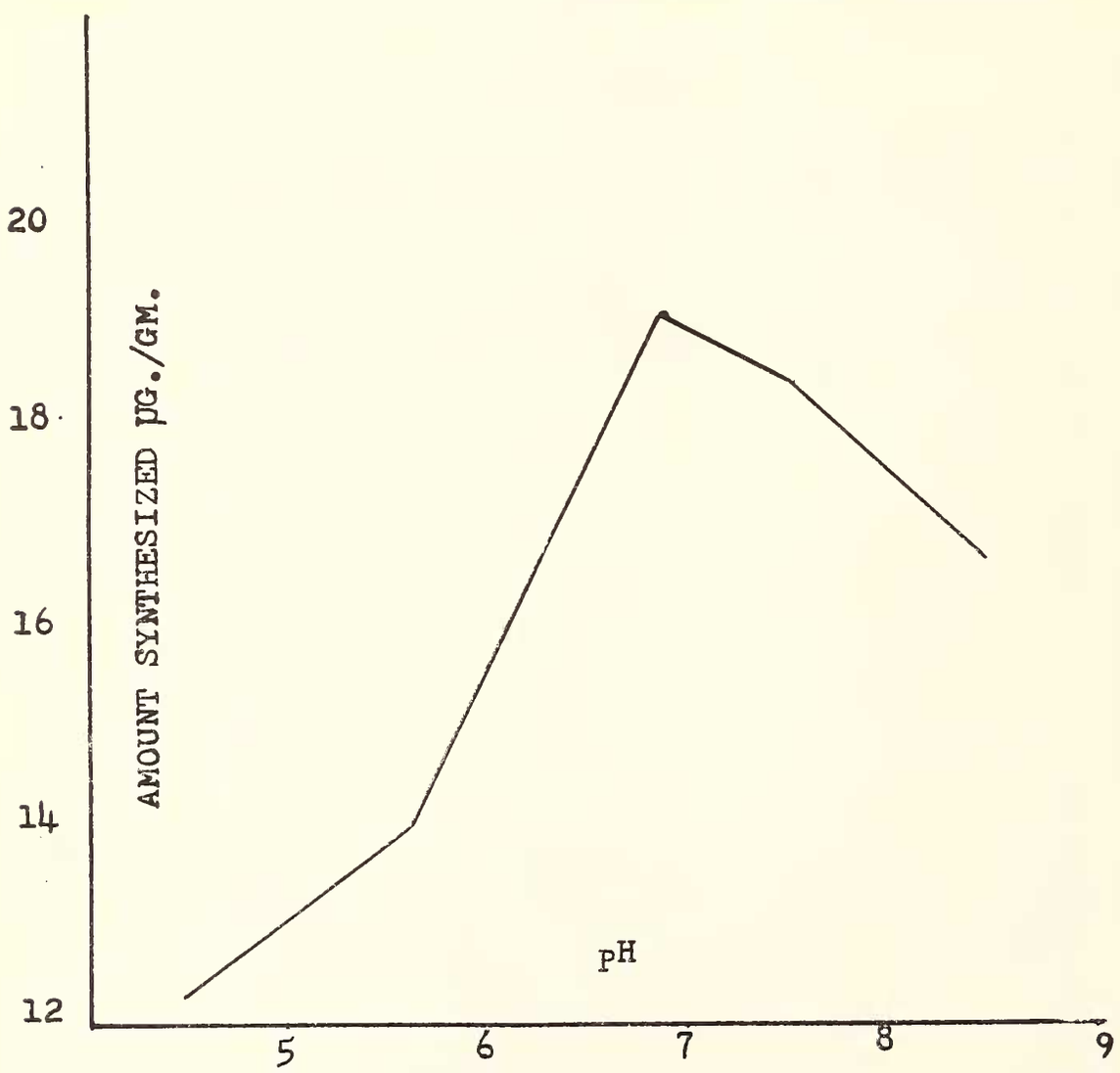

FIG. 1. Effect of pH of the homogenate on the synthesis of ascorbic acid.

* Two means are statistically different when the difference between the means divided by the standard error of the means is equal to two or more.

obtained from mannose and that obtained from the other sugars. Significant differences were also found between xylose and glucose, xylose and fructose, xylose and galactose.

The effect of pH on the synthesis of ascorbic acid is shown in Figure 1. This synthesis is considerably affected by the pH of the homogenate. A value near neutrality appeared to be the most favorable, with an average of 19.0 μg./gm. being formed at a pH of 6.8. An inspection of Figure 1 shows that increasing the alkalinity or acidity resulted in a sharp drop in the amount of

FIG. 2. Effect of concentration of substrate on the synthesis of ascorbic acid.

ascorbic acid formed, with values of 12.2 at a pH of 4.4, and 14.7 at a pH of 8.4.

The concentration of the substrate was also important in determining the ability of insect tissue to synthesize ascorbic acid as shown in Figure 2. Increasing the substrate concentration from 10^{-3} to 5×10^{-3}M resulted in an increase in synthesis, the maximal amount of ascorbic acid being produced at the latter concentration. Figure 2 also shows that an increase in concentra-

tion to 10^{-2}M did not result in any further synthesis. These results indicate that concentrations between 5×10^{-3} and 10^{-2}M are optimal.

Varying the period of incubation resulted in a steady synthesis of ascorbic acid for the first three hours, with an average rate of approximately 6 μg./gm./hr. A continuation of the incubation period to six hours produced a decrease, the rate being

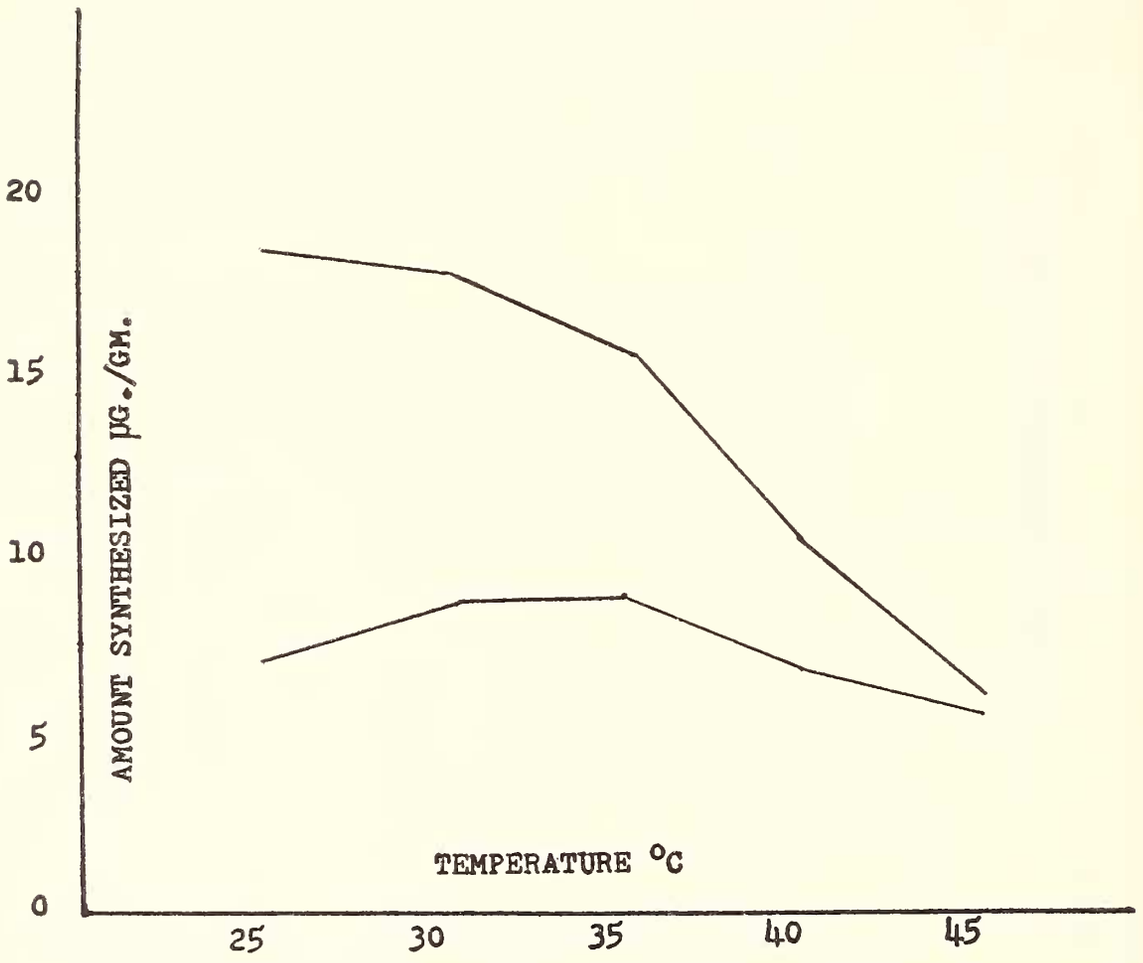

FIG. 3. The effect of the temperature of incubation on the synthesis of ascorbic acid.
Upper graph—three hours incubation
Lower graph—one hour incubation

3 μg./gm./hr.; with nine hours of incubation, it was 1.7, and with fourteen hours, 0.93. Hence the optimal incubation period was three hours.

The temperature of incubation also modifies the amount of ascorbic acid synthesized (Figure 3). Since any determination of optimal temperature must take into account the duration of the test, measurements were after one hour and after three hours

of incubation. In the former case, the differences between the amounts synthesized were not large, 30° C. appearing to be the most favorable temperature. Under these conditions 8.8 μg./gm. were synthesized as compared with 7.2 at room temperature (approximately 25° C.). As the temperature was increased, less ascorbic acid was synthesized. When measurements were made after a three hour incubation period, the level of ascorbic acid production remained almost constant between room temperature and 30° C. Increases in temperature beyond this point resulted in a progressive decrease in synthesis to a low value of 5.0 μg./gm. at 45° C.

Homogenate concentration was found to be important. When it was increased from 10 to 20 per cent the amount of ascorbic acid produced increased from 16.5 to 19.2 μg./gm. A further increase to 30 per cent resulted in a small decrease to 18.5 μg./gm. These results indicate the best homogenate concentration to be 20 per cent.

The addition of manganese ion in the form of $MnCl_2$ did not result in any increased synthesis.

Homogenates of fat bodies produced greater amounts of ascorbic acid than homogenates of whole insects. The amount synthesized was 26.0 as compared with 18.6 μg./gm.

DISCUSSION

In the technique used here, ascorbic acid already present in the tissues and that which may have been in the food in the digestive tract are accounted for by the use of the homogenate blank. However, the blank does not eliminate the possibility that microorganisms could be responsible for its synthesis. Tests using only homogenates of fat bodies greatly reduce the possible role of microorganisms. They do not exclude the possibility that symbiotes present in the mycetocytes of fat bodies could play some part in this synthesis. (Gier, 1936).

The decrease in rate of synthesis when the incubation period was extended beyond three hours or when the temperature was raised above 30° C, may in each case be due to a breakdown of ascorbic acid. Aqueous solutions, and extracts of ascorbic acid, readily undergo oxidation when left exposed to air. The increase in temperature may accelerate oxidation, producing oxalic and

L-threonic acids, compounds which do not couple with the phenylhydrazine reagent. The low values obtained at 40° and 45° C may also be due to thermal inactivation of the enzyme. The temperature of 25° C., optimal in the present experiments, was also found by Gamo and Seki (1954) to be optimal for synthesis of ascorbic acid by the silkworm, *Bombyx mori.*

The lack of increased synthesis when manganese was added to the incubation mixture appears to indicate that manganese is not a necessary cofactor. These results are in agreement with the work of Boyer, Shaw and Phillips (1942) who were unable to substantiate Rudra's (1938) findings that manganese increases the in vitro formation of ascorbic acid when rat and guinea pig liver tissues were supplied with mannose, glucose or galactose.

The ability of insect tissue to utilize sugars in the formation of ascorbic acid agrees with results obtained with vertebrate tissues. Guha and Ghosh (1936) working with spleen, liver and cardiac muscle of rats found that mannose was converted into ascorbic acid. Ruffo and Tartaglione (1948) reported a utilization of mannose, glucose and fructose in the synthesis of ascorbic acid by rat liver and kidney brei. The conversion of glucose to ascorbic acid reported here also agrees with the results obtained with vertebrates. Jackel, Mosback, Burns, and King (1950) as well as Horowitz and King (1953) reported that ascorbic acid was formed when labeled glucose was fed to chloretone-treated rats. However, Smythe and King (1942) found that mannose and glucose were ineffective substrates in the formation of ascorbic acid by rat liver and kidney slices.

The results obtained in this work agree with those of Gamo (1941) who found a synthesis of ascorbic acid during the prepupal and pupal stages of the silkworm, *Bombyx mori.* They also agree with the results of in vitro experiments that Gamo and Seki published in 1954. They reported that the fat bodies of the silkworm were able to convert mannose into ascorbic acid. However, they found that this ability varies considerably during metamorphosis. During the period from the prepupal stage up to the four-day pupae, the amounts synthesized are comparatively great, while negative results were obtained with six-day and eight-day pupae. The synthesis was most intense in prepupae, one-day and two-day pupae. With prepupae they showed that

amounts as great as 97.7 μg./mg. were synthesized; for one-day pupae it was 73.1; and for two-day pupae, 78.3. These values are higher than those obtained in this present work where an average of 26.0 μg./mg. were synthesized by the homogenates of fat bodies. The greatest amount synthesized in any single test of the present experiments was 43.9 μg./gm. The findings of this work also tend to confirm the indirect evidence presented by Wollman, Giroud and Ratismananga (1937) that an enzymatic synthesis of ascorbic acid occurs in the cockroach.

SUMMARY

The ability of the cockroach, *Periplaneta americana* to synthesize ascorbic acid was demonstrated. The monosaccharides mannose, glucose, fructose, galactose and xylose were shown to act as precursors. Of these tested mannose proved to be the best and xylose the poorest substrate. A disaccharide, sucrose, was also utilized in the synthesis of ascorbic acid. Glucoronolactone gave inconclusive results.

The following conditions proved to be most favorable for ascorbic acid synthesis: substrate concentration of 5×10^{-3}M, pH 6.8, three hour incubation period, temperature range of 25°–30° C. and twenty per cent homogenate concentration.

Addition of manganese ion to the incubation mixture did not result in any increase in the synthesis.

Homogenates of fat bodies synthesized greater amounts of ascorbic acid than whole homogenates.

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